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Adaptation of the Highly Productive T7 Expression System to *Streptomyces lividans*▽†

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Streptomyces lividans **is a Gram-positive bacterium known for its remarkable secretion efficiency and low extracellular protease activity. In the present work, we adapted the highly productive T7 expression system to *S. lividans*. A codon-optimized T7 RNA polymerase gene was chromosomally integrated, and a bifunctional T7 expression vector was constructed.**

The *Escherichia coli* T7 RNA polymerase (T7 RNApol)-based expression system, developed by Studier and Moffatt (35), is currently used in many laboratories for heterologous protein production. The system is based on the T7 bacteriophage RNA polymerase, which directs selective transcription of genes cloned downstream of the major T7 late promoter. T7 RNApol is characterized by a very high activity, elongating chains about five times faster than *E. coli* RNA polymerase, and can generate very long mRNAs (19, 35). Although *E. coli* has proved to be useful for gene overexpression, different problems can occur and limit the productivity (38). As such, the use of an alternative expression host is often desirable to obtain adequate protein production.

Streptomycetes are Gram-positive G+C-rich bacteria known for their high secretion capacity and have been used extensively in commercial settings for antibiotic production in very-large-scale fermentation systems (6). Among the streptomycetes, the readily transformable *Streptomyces lividans* has been used for the expression of a wide variety of genes from diverse sources (4). This host can secrete directly in the culture medium large quantities of proteins in mature conformation, and given that few endogenous proteins are present in the medium, downstream purification processes are simplified. *S. lividans* also displays a very low level of endogenous extracellular protease activity, making it a suitable host for heterologous protein production (26). Most of the *S. lividans* expression systems are based on strong constitutive promoters (4, 34). Few inducible promoters are also used, the thiostrepton-inducible *tipA* promoter (*tipAp*) being the most popular (37).

Since the original publication of the *E. coli* T7 expression system, it has been adapted to mammalian cells and several bacteria (2, 9, 13, 16, 18, 22, 25). In this report, the T7 expression system was adapted to *S. lividans* to combine the T7 RNApol efficiency with the great features of this host. During the revision process of this paper, we learned that a similar system had been developed and published in a thesis at the University of Stuttgart (17).

T7 RNA polymerase production in *S. lividans*. To efficiently express the T7 RNApol gene in *S. lividans*, the four rare TTA_{leu} codons were replaced by CTC_{leu} codons by overlap extension PCR (20) (see the supplemental material). The production of the T7 RNApol was evaluated with the codon-modified (CM) and wild-type (W) genes. They were cloned into the pIJ702-derived (24) multicopy expression vector pIAFC109 (François Shareck, personal communication), under the control of the constitutive promoter C109, resulting in pIAFC109_T7CM and pIAFC109_T7W. Both constructs were introduced in *S. lividans* 10-164 (21) by protoplast transformation according to Kieser et al. (26). Protein production and mycelium disruption were conducted as described by Nisole et al. (31). The intracellular protein fractions were analyzed by Coomassie-stained SDS-PAGE and Western blotting (Fig. 1). The T7 RNApol-producing strain *E. coli* BL21/pAR1219 (10) was used as a positive control. Western blotting was performed with anti-T7 RNA polymerase mouse monoclonal antibody (Novagen) and alkaline phosphatase-conjugated goat anti-mouse antibody (GE Healthcare). Colorimetric detection was performed with the substrates nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Bio-Rad). On SDS-PAGE, a band of about 100 kDa appeared in the intracellular fraction of 10-164/pIAFC109_T7CM, but was undetectable in 10-164/pIAFC109_T7W. Western blot analysis confirmed that T7 RNApol was produced with the codon-modified gene but not detected with the wild-type version.

In *Streptomyces lividans*, TTA_{leu} is the rarest codon (Codon Usage Database; www.kazusa.or.jp/codon/) and can be efficiently transcribed by only one tRNA encoded by *bldA* (28, 39). The *bldA* gene is constitutively transcribed, but the mature form of the tRNA seems to be present only near the end of the logarithmic growth phase (29), when the secondary metabolism is activated (7). Different papers have shown that the presence of a TTA_{leu} codon in a gene causes temporal expression delay or prevents translation in a *bldA* mutant strain (23, 29, 32, 33). Since the T7 RNApol gene contains four TTA_{leu} codons, it was not surprising that its expression was impaired in *S. lividans*. By mutating these four codons, the T7 RNApol gene was overexpressed for the first time in *S. lividans*.

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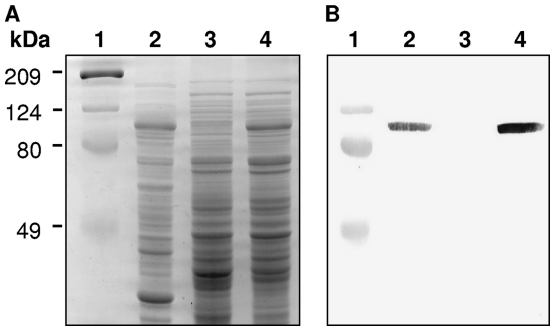

FIG. 1. Analysis of T7 RNApol production by Coomassie-stained SDS-PAGE (A) and Western blotting (B). M, molecular mass standard; lane 1, positive control; lane 2, *S. lividans* 10-164/pIAFC109_T7W; lane 3, *S. lividans* 10-164/pIAFC109_T7CM.

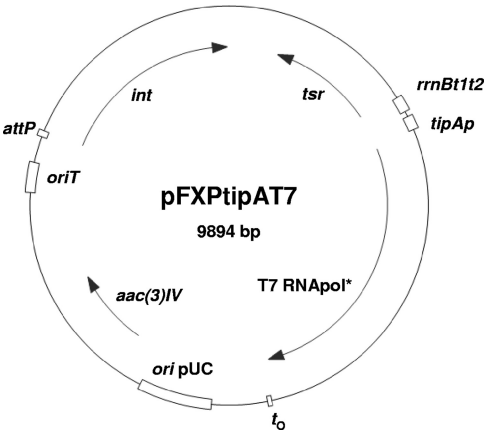

FIG. 2. Structure of the integrative plasmid pFXPtipAT7. T7 RNApol*, codon-modified gene of the T7 RNA polymerase; *tipAp*, thiostrepton-inducible promoter; *aac(3)IV*, apramycin acetyltransferase, apramycin resistance; *tsr*, 23S A^{1067} rRNA methylase, thiostrepton resistance; *int*, ϕC31 integrase; *rrnBt1t2*, transcription terminators T1 and T2 from *E. coli rrnB* gene; t_O, λ transcription terminator; *ori* pUC, replication origin of pUC18; *oriT*, RK2 origin of transfer; *attP*, ϕC31 phage attachment site.

S. *lividans* T7 expression strain. The codon-modified T7 RNApol gene was cloned into a derivative of pSET152 (3) under the control of the thiostrepton-inducible promoter *tipAp* (30), resulting in pFXPtipAT7 (Fig. 2) (for detailed construction, see the supplemental material). This construct was integrated into the chromosome of *S. lividans* 10-164 via the bacteriophage ϕC31 *att*/*int* system (3), giving rise to the apramycin- and thiostrepton-resistant *S. lividans* 10T7 strain. Integration at the chromosomal *attB* site was confirmed by PCR and DNA sequencing, but T7 RNApol production by *S. lividans* 10T7 under the thiostrepton-induced condition could not be detected by SDS-PAGE or Western blotting (results not shown). Strain 10T7 has not shown any growth retardation in the presence of thiostrepton compared to that of a noninduced culture (results not shown). It has to be noted that pSET152-derived plasmids can sometimes integrate as a tandem repeat and into at least three pseudo-*attB* sites with a 300-fold lower efficiency (8). The plasmid pFXPtipAT7 can be used to create T7 expression strains in *S. lividans* and *Streptomyces coelicolor* with one simple transformation step (8, 37).

Bifunctional T7 expression vector. Construction of pFX583 was realized by using the *E. coli* T7 expression vector pET-9a (Novagen) (36) as the backbone (Fig. 3). Detailed construction is presented in the supplemental material. The pFX583 vector contains pMB1 (5) and pJV1 (1) replicons, allowing replication in *E. coli* and *S. lividans* with a high copy number. Kanamycin or neomycin selection can also be used with both bacteria due to the FD Neo-S cassette (11). The vector pFX583 is compatible with the widely used *Streptomyces* pIJ101 replicon (1). Shuttle vectors are very attractive because they allow performance of all the DNA manipulation in *E. coli* but are sometimes structurally unstable in *Streptomyces* for unknown reasons (26). Here, pFX583 has been maintained in *E. coli* and *S. lividans* in the presence of selection without notable structure instability. Since pFX583 harbors an *oriT* sequence, it can be transferred by conjugation from *E. coli* to *Streptomyces* strains that are difficult to transform. The presence of a λ *cos* sequence allows the use of pFX583 as a cosmid vector for large DNA fragment cloning.

T7 RNA polymerase-directed xylanase production. The efficacy of the *S. lividans* T7 expression system was demonstrated by overexpressing the gene encoding a truncated version of the *S. lividans* xylanase A (*xlnA2*) (12). The *xlnA2* gene was amplified by PCR and cloned into the NdeI and BamHI sites of pFX583. The resulting construct, pFX583xlnA2, was introduced into the *S. lividans* T7 expression strain 10T7 by protoplast transformation. Transformants were picked from R5 medium (26) and streaked onto Bennett agar (26) containing 50 μg/ml apramycin and 50 μg/ml kanamycin. After 3 to 4 days of incubation at 34°C, the sporulated mycelium was used to inoculate tryptic soy broth medium (Difco) and cultured in an Erlenmeyer flask. Incubation was carried out at 34°C on a rotary shaker at 240 rpm for 48 h. Recombinant expression of *xlnA2* was induced by addition of thiostrepton to the culture medium. Different concentrations of thiostrepton were tested, and 25 μg/ml allowed the highest XlnA2 production (see Fig. S1 in the supplemental material). Maximal enzyme production was obtained when thiostrepton was added at the beginning of the incubation period, and significant increase in activity stopped after 48 h (see Fig. S2 in the supplemental material).

Equal volumes of culture supernatants were analyzed by Coomassie-stained SDS-PAGE, and xylanase activity was measured as described by Ebanks et al. (15) (Fig. 4). Xylanase A2 was absent in noninduced cultures, while readily detected as a 31-kDa band by SDS-PAGE in the presence of thiostrepton. As for all *tipAp*-based expression systems, thiostrepton also induced the production of the TipAL protein that can be seen on SDS-PAGE around 20 kDa (30). Xylanase activity assays were consistent with SDS-PAGE analysis. After 48 h, no xylanase activity was measured in noninduced cultures, while 13.8 U/ml (30.2 U/mg) was detected under induced conditions, clearly demonstrating inducible *xlnA2* expression in *S. lividans* 10T7. Based on the specific activity of the purified XlnA2 (286 U/mg) (15), the concentration can be estimated to be 48 mg/liter.

To confirm that pFX583 is also functional in *E. coli*, pFX582xlnA2 was introduced into the T7 expression strain BL21(DE3) (Novagen). Protein production was induced with 0.025 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and

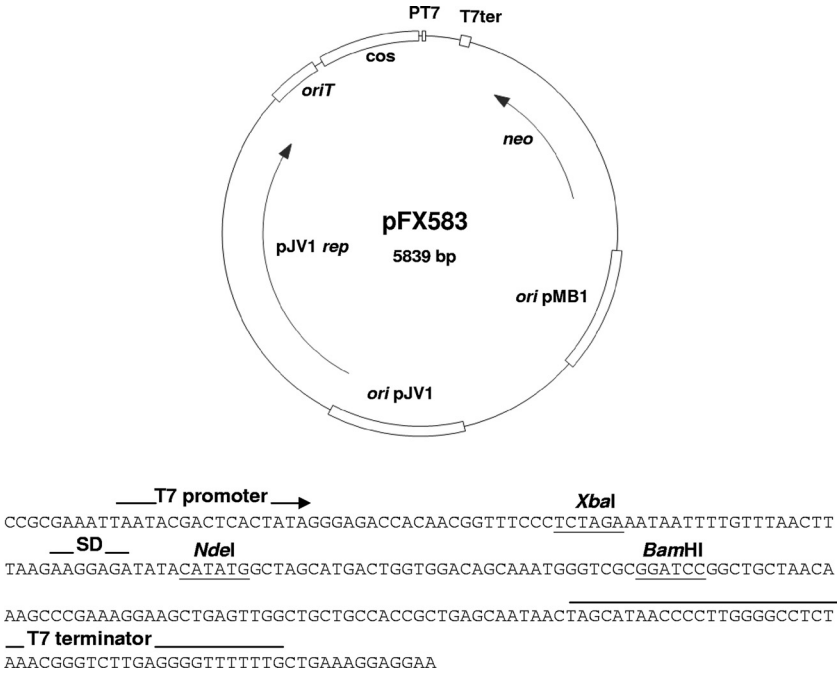

FIG. 3. Structure of the bifunctional T7 expression vector pFX583. PT7, T7 gene ϕ10 promoter; T7ter, T7 transcription terminator ϕ*t*; *neo*, Tn*903* aminoglycoside phosphotransferase, kanamycin and neomycin resistance; *ori* pMB1, replication origin of pMB1; *ori* pJV1, replication origin of pJV1; pJV1 *rep*, pJV1 Rep protein; *oriT*, RK2 origin of transfer; cos, λ cohesive end site; SD, Shine-Dalgarno sequence.

conducted for 20 h at 37°C. Supernatants of induced and noninduced cultures were analyzed by Coomassie-stained SDS-PAGE (see Fig. S3 in the supplemental material). As for *S. lividans*, the xylanase A2 was produced under induced conditions without obvious expression leaking in the absence of IPTG.

Conclusion. Combined with the bifunctional T7 expression vector pFX583, *S. lividans* 10T7 allowed inducible T7 RNApol-directed overproduction of the xylanase A2 without detectable expression leaking in the absence of inducer. Although the amount of protein produced was relatively low compared to what can be obtain with noninducible *Streptomyces* expression systems (14, 27, 31), the T7 expression system developed here presents interesting features. It is well regulated, has the potential to transcribe very large DNA fragments, and can be used in combination with pIJ101-derived plasmids. The vector pFX583 is functional in *E. coli* and *Streptomyces* strains producing T7 RNApol. With a single construction, it is therefore possible to compare the expression of a gene in two kinds of hosts and determine which one is the most appropriate based on the productivity and requirements of the study.

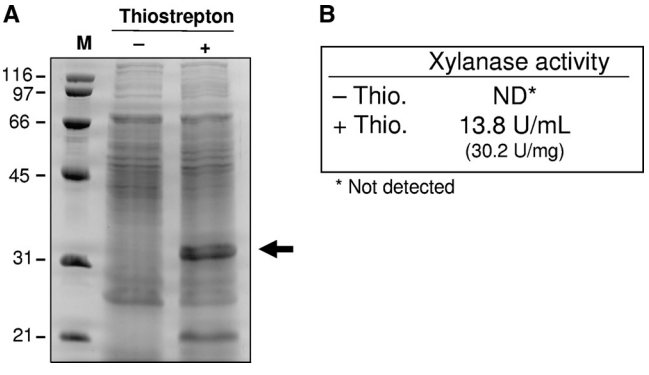

FIG. 4. (A) Coomassie-stained SDS-PAGE showing the extracellular production of XlnA2 in noninduced (−) and induced (+) cultures. M, molecular mass standard. (B) Xylanase activity in noninduced (− Thio.) and induced (+ Thio.) cultures.

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